

# Molecular bases of cyclodextrin adapter interactions with engineered protein nanopores

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**Engineered protein pores have several potential applications in biotechnology: as sensor elements in stochastic detection and ultrarapid DNA sequencing, as nanoreactors to observe single-molecule chemistry, and in the construction of nano- and micro-devices. One important class of pores contains molecular adapters, which provide internal binding sites for small molecules. Mutants of the  $\alpha$ -hemolysin ( $\alpha$ HL) pore that bind the adapter  $\beta$ -cyclodextrin ( $\beta$ CD)  $\sim 10^4$  times more tightly than the wild type have been obtained. We now use single-channel electrical recording, protein engineering including unnatural amino acid mutagenesis, and high-resolution x-ray crystallography to provide definitive structural information on these engineered protein nanopores in unparalleled detail.**

alpha-hemolysin | single molecule | stochastic sensing | structure | unnatural amino acid

Many research groups have used protein engineering to obtain enzymes and antibodies with new properties suited for specific tasks (1–6). Fewer groups have taken on the difficult problem of engineering membrane proteins (7). We have engineered the  $\alpha$ -hemolysin protein pore, mindful of several potential applications in biotechnology, including its ability to act as a detector in stochastic sensing (8) and ultrarapid DNA sequencing (9), to serve as a nanoreactor for the observation of single-molecule chemistry (10) and to act as a component for the construction of nano- and microdevices (11).

An important breakthrough in this area, which enabled the stochastic sensing of organic molecules including the detection of DNA bases in the form of nucleoside monophosphates (12, 13), was the discovery of internal molecular adapters, a form of non-covalent protein modification (14). Most useful have been cyclodextrin (CD) adapters, which have until now been used in the absence of detailed structural information about how they work. The present paper is a definitive investigation, which provides such information through the application of a wide variety of technical approaches: single-channel electrical recording, protein engineering including unnatural amino acid mutagenesis, and x-ray crystallography. The studies employing mutagenesis show that the striking interactions seen in the crystal structures also occur in individual pores in lipid bilayers.

We reveal that the tight-binding  $\alpha$ HL mutants (15) M113N<sub>7</sub> and M113F<sub>7</sub> bind  $\beta$ CD in different orientations within the heptameric pore. In the case of M113N<sub>7</sub>, the top (primary hydroxyls) of the CD ring faces the trans entrance of the pore. In the case of M113F<sub>7</sub>, the bottom (secondary hydroxyls) of the CD ring faces the trans entrance, while the top of the ring is bonded to the pore through remarkable CH- $\pi$  interactions. Another tight-binding mutant, M113V<sub>7</sub>, can bind the CD in both orientations. These results illustrate the exquisite level of engineering that can be achieved with protein nanopores, which is, for example, far beyond what is possible with solid-state pores. The work also provides information valuable for the design of new binding sites

within the lumen of the  $\alpha$ HL pore or within other  $\beta$ -barrel proteins. Our results will be of interest to others exploring the interactions of CDs with the  $\alpha$ HL pore (16, 17), including groups involved in computational studies (18, 19). In addition CDs bind to a variety of other pores, including porins (20, 21) and connexins (22), and are being tested in vivo as blockers of the anthrax protective antigen pore (23, 24). The CD adapter concept has also been incorporated into other formats, e.g., with glass nanopores (25), and artificial pores based on CDs have been made by several groups (26–28). Our work is pertinent to these studies.

## Results

**Kinetics and Thermodynamics of the Interactions of  $\beta$ CD with  $\alpha$ HL Pores Containing Met, Phe and Asn at Position 113.** We showed earlier that position 113 in the  $\alpha$ HL pore (Fig. 1A) is critical for the binding of  $\beta$ CD (14). Subsequently, residue 113, which is Met in the WT protein, was changed to each of the remaining 19 naturally occurring amino acids by site-directed mutagenesis (15). We found that 11 of these mutants, expressed as homoheptamers, bound  $\beta$ CD with a similar affinity and with similar kinetics to the WT homoheptamer. Two mutants (P, W) bound  $\beta$ CD about 10 times more strongly than the WT homoheptamer, while six of them (V, H, Y, D, N, F) bound with high affinity, i.e., with a  $K_d$  value  $10^3$  to  $10^4$  times lower than the WT.

Remarkably, the side chains of the latter six amino acids bear little resemblance to one another, and this issue is addressed in the present paper. We first examined the two amino acids with the most disparate side chains (F and N) by making heteromeric pores containing WT (Met-113), M113F, and M113N subunits. Three series of heteroheptamers were produced: WT<sub>7-n</sub>M113N<sub>n</sub>, WT<sub>7-n</sub>M113F<sub>n</sub>, and M113F<sub>7-n</sub>M113N<sub>n</sub>. The heteroheptamers were separated by SDS-polyacrylamide gel electrophoresis aided by an oligoaspartate (D8) tail on the first of the two types of subunit (Fig. 1B) (29). All 21 combinations of WT, M113F, and M113N subunits formed  $\alpha$ HL pores that interacted with  $\beta$ CD as shown by single-channel current recordings, which revealed the extent of block by  $\beta$ CD (Fig. S1), the association and dissociation

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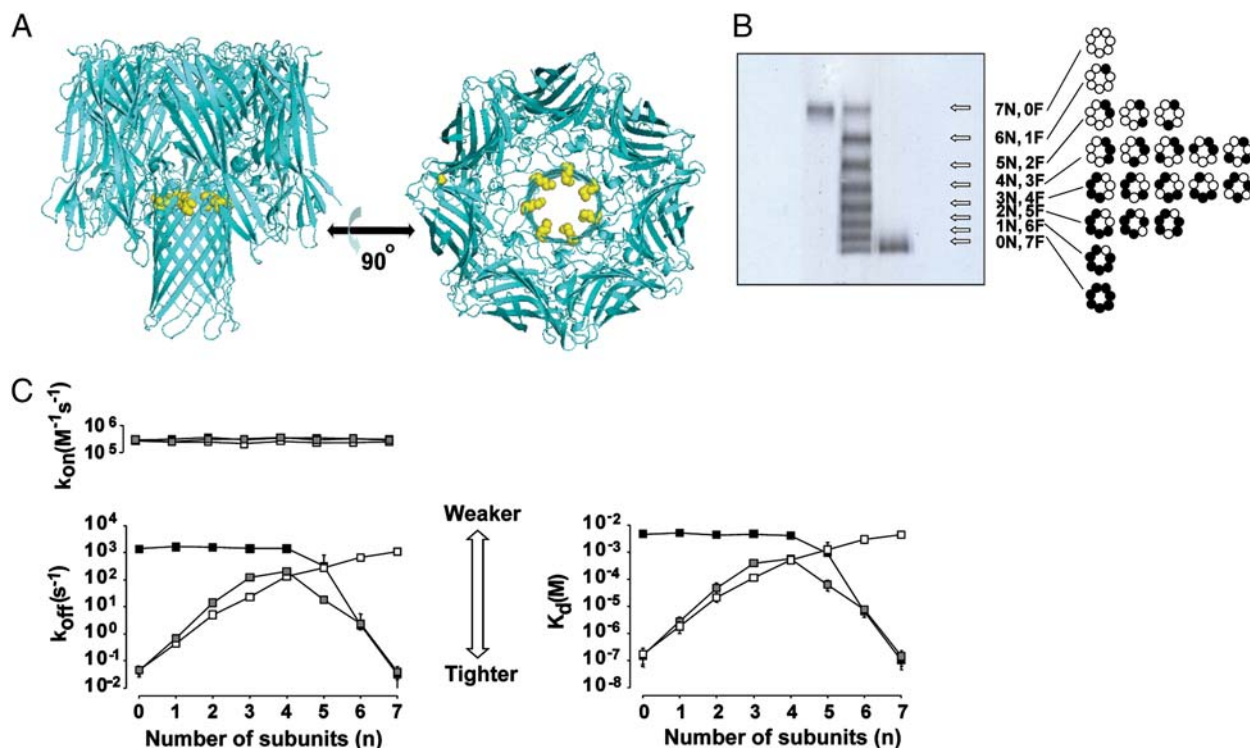
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**Fig. 1.** Binding of  $\beta$ CD by heteromeric  $\alpha$ HL pores formed by WT, M113F and M113N subunits. (A) Crystal structure of WT- $\alpha$ HL (61) showing residue 113 (Met, yellow). Left panel, side view and right panel, top view. (B) Separation of  $^{35}\text{S}$ -labeled  $\alpha$ HL heteroheptamers by SDS-polyacrylamide electrophoresis. The separation of the M113F $_{7-n}$ M113N $_n$  heteromers is shown as detected by autoradiography of a dried gel. The M113F subunits carried a D8 tail. Lane 1, M113N $_7$ ; lane 2, M113F $_{7-n}$ M113N $_n$  (the heteromers formed from several preparations made with differing ratios of M113F and M113N subunits were mixed to give roughly equal amounts of each subunit combination); lane 3, M113F $_7$ . A diagram of the eight different combinations of subunits and their permutations is shown to the right of the autoradiogram. The various permutations are not separated by electrophoresis. (C) Kinetics of the interaction of  $\beta$ CD with single heteromeric  $\alpha$ HL pores as determined by bilayer recording. Values of  $k_{\text{on}}$  were calculated by using  $k_{\text{on}} = 1/(\tau_{\text{on}}[\beta\text{CD}])$ , where  $\tau_{\text{on}}$  is the mean interevent interval. Values of  $k_{\text{off}}$  were determined by using  $k_{\text{off}} = 1/\tau_{\text{off}}$ , where  $\tau_{\text{off}}$  is the mean dwell time of  $\beta$ CD in the pore. Values of  $K_d$  were calculated by using  $K_d = k_{\text{off}}/k_{\text{on}}$ . Each point represents the mean  $\pm$  s.d. for three or more determinations. Where they cannot be seen, the s.d. values lie within the symbol. Black squares, WT $_{7-n}$ M113N $_n$ ; gray squares, M113F $_{7-n}$ M113N $_n$ ; empty squares, M113F $_{7-n}$ WT $_n$ .

rate constants for  $\beta$ CD ( $k_{\text{on}}$  and  $k_{\text{off}}$ ), and (from the latter) the equilibrium dissociation constant for  $\beta$ CD ( $K_d = k_{\text{off}}/k_{\text{on}}$ ) (15).

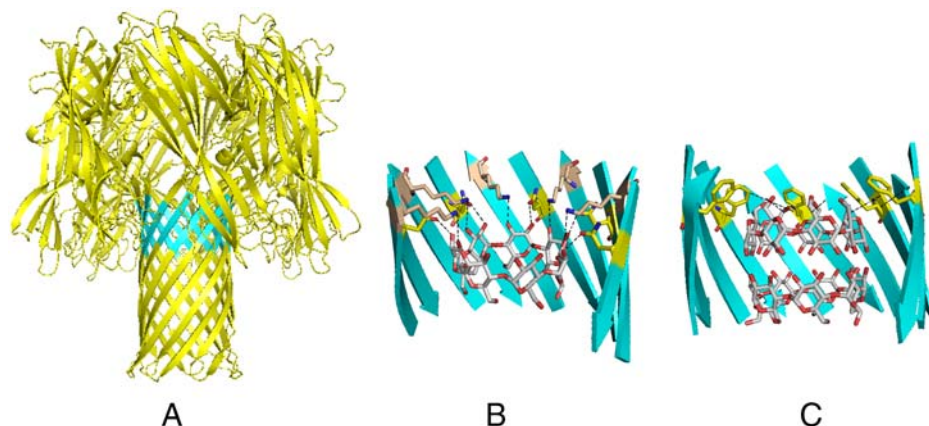
The  $k_{\text{on}}$  values for  $\beta$ CD for the 21 combinations of subunits were all similar at  $\sim 5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  (Fig. 1C, Upper). By contrast, the  $k_{\text{off}}$  values differed widely, ranging from  $\sim 5 \times 10^{-2} \text{ s}^{-1}$  to  $\sim 10^3 \text{ s}^{-1}$ . For WT $_{7-n}$ M113N $_n$  and WT $_{7-n}$ M113F $_n$ , the  $k_{\text{off}}$  values decreased as M113N or M113F subunits were added. In the case of M113N, there was a steep drop in the value of  $k_{\text{off}}$  after the fifth subunit had been incorporated. In the case of M113F, the decrease in the value of  $k_{\text{off}}$  occurred less precipitously as the M113F subunits were added (Fig. 1C, Lower). Intriguingly, with M113F $_{7-n}$ M113N $_n$ ,  $k_{\text{off}}$  first increased as M113N subunits were added to M113F $_7$  until  $n = 4$  (M113F $_3$ M113N $_4$ ) and then decreased for larger values of  $n$  (Fig. 1C, Lower). We recognize that there is more than one permutation of heteromers containing two to five mutant subunits (Fig. 1B), but we have ignored this fact here because no significant differences in the properties of individual heteromers were observed. For example, 42 recordings were made of WT $_5$ M113N $_2$ , which has three permutations. Because,  $k_{\text{on}}$  showed little variation with subunit composition, the variation in  $K_d$  was similar to the variation in  $k_{\text{off}}$  (Fig. 1C).

While these studies were in progress, the crystal structures of  $\beta$ CD complexed to M113N $_7$  (Fig. 2B) and M113F $_7$  (Fig. 2C) were solved (Table S1) (30). High-resolution structures could be obtained because the CD and the  $\alpha$ HL pore have the same  $C_7$  symmetry. In the case of M113N $_7$ ,  $\beta$ CD is bound with the secondary hydroxyl face “upward” (Fig. 2B). In each glucose unit of the  $\beta$ CD, the 2-hydroxyl is hydrogen bonded to the side-chain amide of an Asn-113 (the residue introduced by mutagenesis) and the

3-hydroxyl is hydrogen bonded to the  $\epsilon$ -amino group of Lys-147. In the case of M113F $_7$ , two  $\beta$ CDs are bound to the  $\alpha$ HL pore (Fig. 2C). It is the top  $\beta$ CD in the structure that concerns us, because it is in contact with the Phe-113 residues introduced by mutagenesis. It is immediately apparent that the top  $\beta$ CD in M113F $_7$  is in the opposite orientation to the  $\beta$ CD in M113N $_7$  with each 6-hydroxyl group in a CH- $\pi$  bonding interaction (31–35) with a Phe-113 side chain. The opposite orientations of the  $\beta$ CDs in M113N $_7$  and M113F $_7$  immediately explain why heteromers formed from similar numbers of M113N and M113F subunits (e.g., M113N $_4$ M113F $_3$ ) bind  $\beta$ CD weakly (see also Discussion).

**Unnatural Amino Acid Mutagenesis.** To further explore the range of noncovalent interactions that are available when  $\beta$ CD binds to the  $\alpha$ HL pore, five unnatural amino acids (Fig. 3A and Fig. S2) were incorporated at position 113, by using the in vitro nonsense codon suppression method (36). In particular, we had noted that M113V $_7$  containing the  $\beta$ -branched Val binds  $\beta$ CD tightly (15), and therefore we compared cyclopropylglycine (Cpg) and cyclopropylalanine (Cpa). We also further examined the means by which M113F $_7$  binds  $\beta$ CD tightly, by comparing the properties of 4-fluorophenylalanine (f1F), pentafluorophenylalanine (f5F), and cyclohexylalanine (Cha) at position 113.

The five homomeric pores all produced single-channel currents with unitary conductance values in the range expected for properly assembled heptamers (Fig. S3). All five bound  $\beta$ CD (Fig. 3B, Level 2), either tightly (f1F, Cpg) or weakly (f5F, Cha, Cpa) as described in detail below. During the long  $\beta$ CD binding events, additional current spikes were seen (Fig. 3B). Similar



**Fig. 2.** X-ray structures of M113N and M113F homoheptamers with  $\beta$ CD bound. (A) Side view of heptameric  $\alpha$ HL.  $\beta$ CD binds in the blue highlighted region. (B)  $\beta$ CD bound to M113N<sub>7</sub> (dotted lines indicate hydrogen bonding). The side chains of Lys-147 are in pale brown and the side chains of Asn-113 are in yellow. (C)  $\beta$ CD bound to M113F<sub>7</sub> (dotted lines indicate CH- $\pi$  bonding). The side chains of Phe-113 are in yellow. The second  $\beta$ CD in the M113F<sub>7</sub> · ( $\beta$ CD)<sub>2</sub> structure is hydrogen bonded to the top  $\beta$ CD in a head-to-head arrangement and has no apparent interactions with the protein. For both (B) and (C), four  $\beta$  strands were omitted from the barrel to give a better view.

events had been observed previously with certain Met-113 replacement mutants and may represent movement of the  $\beta$ CD at its binding site (e.g., rotation about axes perpendicular to the C<sub>7</sub> axis) (15). The additional current spikes were more prevalent for M113V<sub>7</sub> and M113Cpg<sub>7</sub>, which may take part in more conformationally labile interactions with  $\beta$ CD, compared with say M113F<sub>7</sub> (Fig. S4).

**Interactions of  $\beta$ CD with Homoheptamers Bearing Aromatic Residues at Position 113.** To further understand the nature of the binding of  $\beta$ CD to aromatic side chains, we examined the kinetics of  $\beta$ CD binding to the homoheptamers containing f1F or f5F at position 113, M113f1F<sub>7</sub> and M113f5F<sub>7</sub> (Fig. 3C). For both mutants, the value of  $k_{on}$  was very similar to that of WT<sub>7</sub>, but the values of  $k_{off}$  and therefore  $K_d$  for M113f1F<sub>7</sub> differed dramatically from WT<sub>7</sub> and were close to the values for the tight-binding mutant M113F<sub>7</sub> (Table S24). By contrast,  $k_{off}$  and  $K_d$  for M113f5F<sub>7</sub> were similar to the values for WT<sub>7</sub> (Table S24).

To determine whether M113f1F<sub>7</sub> binds  $\beta$ CD in the same orientation as M113F<sub>7</sub> (Fig. 2C), we made heteromers of the M113f1F subunit with M113N or M113F and examined M113F<sub>4</sub>M113f1F<sub>3</sub> and M113N<sub>4</sub>M113f1F<sub>3</sub>. M113F<sub>4</sub>M113f1F<sub>3</sub> binds  $\beta$ CD as strongly as either M113F<sub>7</sub> or M113f1F<sub>7</sub>, but M113N<sub>4</sub>M113f1F<sub>3</sub> binds  $\beta$ CD weakly with a similar affinity to WT<sub>7</sub> (Fig. 3D and Table S3). Therefore, it is reasonable to infer that M113F<sub>7</sub> and M113f1F<sub>7</sub> bind  $\beta$ CD in the same orientation with the 6-hydroxyl groups of the CD in proximity to the aromatic rings on the protein.

Cyclohexylalanine (Cha) was used to replace the aromatic side chains with a roughly isosteric hydrophobic group. Again the value of  $k_{on}$  for  $\beta$ CD was little changed, but  $k_{off}$  for M113Cha<sub>7</sub> had an intermediate value of  $42 \pm 6$  s<sup>-1</sup>. Therefore, M113Cha<sub>7</sub> binds  $\beta$ CD more weakly than M113F<sub>7</sub> but distinctly more strongly than the WT<sub>7</sub> pore (Table S24 and Fig. 3C).

**Interactions of  $\beta$ CD with Homoheptamers Bearing Hydrophobic Residues at Position 113.** M113V<sub>7</sub> binds  $\beta$ CD very strongly, and therefore we compared  $\alpha$ HL pores with Cpg or Cpa at position 113. Cpg is roughly isosteric with Val, and like Val has a  $\beta$ -branched side chain. Gratifyingly, M113Cpg<sub>7</sub> has a  $k_{on}$  value similar to the other  $\alpha$ HL pores, and  $k_{off}$  and  $K_d$  values close to those of M113V<sub>7</sub> (Table S2B and Fig. 3E). Cyclopropylalanine (Cpa), with an additional methylene group compared to Cpg, is roughly isosteric with Leu, a weak binder, and M113Cpa<sub>7</sub> also binds  $\beta$ CD weakly with  $k_{on}$ ,  $k_{off}$  and  $K_d$  values similar to those of WT<sub>7</sub> (Table S2B and Fig. 3E). M113I<sub>7</sub> and M113T<sub>7</sub>, which are  $\beta$ -branched, are also weak binders, but Ile and Thr are less closely related to Val than Cpg.

To determine whether M113V<sub>7</sub> binds  $\beta$ CD in the same orientation as M113F<sub>7</sub> or M113N<sub>7</sub> (Fig. 2), we made heteromers of

M113V and the M113N or M113F subunits. M113V<sub>3</sub>M113F<sub>4</sub>, M113V<sub>4</sub>M113F<sub>3</sub>, M113V<sub>3</sub>M113N<sub>4</sub>, and M113V<sub>4</sub>M113N<sub>3</sub> were examined in detail. All four heteroheptamers bound  $\beta$ CD more weakly than M113V<sub>7</sub>, M113F<sub>7</sub> or M113N<sub>7</sub> (Fig. 3F and Table S4), suggesting that Val at position 113 interacts with  $\beta$ CD strongly but in a different manner to either Phe or Asn. Each heteromer exhibited a range of  $K_d$  values, perhaps reflecting the various possible permutations of the two different subunits around the central axis of the heptamer, although this heterogeneity was not seen for heteromers made from WT, M113F and M113N (Fig. 1).

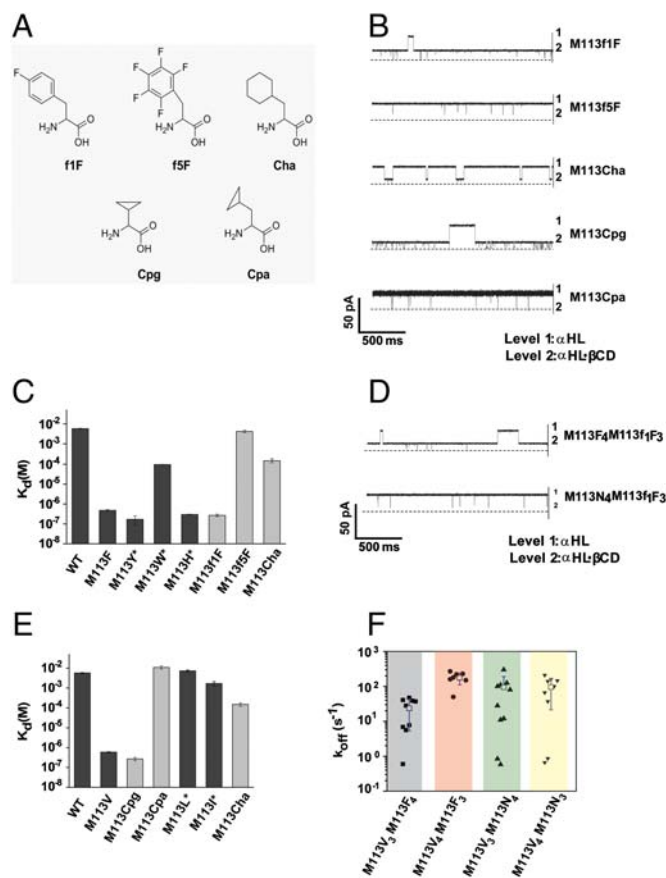
## Discussion

Soon after we discovered that  $\beta$ CD binds to the WT- $\alpha$ HL pore for around a millisecond, we found a mutant pore, M113N<sub>7</sub>, that releases  $\beta$ CD  $\sim 10^4$  times more slowly (14). This prompted us to examine all 19 mutants in which residue 113 is replaced by a natural amino acid, with the surprising result that a collection of amino acids with structurally unrelated side chains (V, H, Y, D, N, F) are tight binders (15). Here, we have examined the nature of the binding interactions more closely by single-channel electrical recording, protein engineering including unnatural amino acid mutagenesis, and high-resolution x-ray crystallography, and we provide the first definitive structural information on an engineered protein nanopore.

We find that  $\beta$ CD can bind tightly to the  $\alpha$ HL pore in three different ways depending on the residue at 113, as exemplified by Asn, Phe, and Val. Because Asn and Phe have quite different side chains, we first compared the ability of M113N and M113F subunits to take part in binding the CD. The examination of heteromeric proteins containing WT (Met-113), M113N and M113F subunits showed that the replacement of WT subunits in WT<sub>7</sub> with M113N or M113F subunits led to increased affinity for  $\beta$ CD. The more M113N or M113F subunits that were added, the tighter binding became. By contrast, when subunits in M113N<sub>7</sub> were replaced with M113F subunits, binding became weaker, reaching a minimum at three to four M113F subunits, and then increasing in strength with five M113F subunits or more (Fig. 1C). Parallel structural studies (30) revealed the basis of the “opposing” effects of the M113N and M113F subunits.  $\beta$ CD binds to M113N<sub>7</sub> in the opposite orientation to that in which it binds to M113F<sub>7</sub>. In M113N<sub>7</sub>, the secondary hydroxyls in the  $\beta$ CD ring are hydrogen bonded to Lys-147 and Asn-113 (Fig. 24). By contrast,  $\beta$ CD interacts with M113F<sub>7</sub> through its primary hydroxyl face (Fig. 2B).

It seemed likely that M113V<sub>7</sub> bound  $\beta$ CD in yet another way, and this was examined by forming heteromers between M113V and M113N or M113F. The presence of three or four subunits of either M113N or M113F greatly decreases the affinity of the pore for  $\beta$ CD (Fig. 3F), with an average  $k_{off}$  of  $7.3 \times 10^1$  s<sup>-1</sup>, indicating that a third binding mode is indeed operating





**Fig. 3.** Properties of pores containing natural and unnatural amino acid substitutions at position 113. The data were recorded at +40 mV in 1.0 M NaCl, 10 mM sodium phosphate, pH 7.5. (A) Unnatural amino acids used in this study: 4-fluorophenylalanine, f1F; pentafluorophenylalanine, f5F; cyclohexylalanine, Cha; cyclopropylglycine, Cpg; cyclopropylalanine, Cpa. (B) Representative current traces from single-channel recordings of  $\beta$ CD binding to M113F<sub>4</sub>M113F<sub>1</sub>F<sub>3</sub> and M113N<sub>4</sub>M113F<sub>1</sub>F<sub>3</sub>.  $\beta$ CD (40  $\mu$ M final) was added to the trans chamber. The broken line indicates zero current. (C) Interaction of  $\beta$ CD with homomeric  $\alpha$ HL pores containing aromatic amino acids at position 113.  $K_d$  values for the interaction between  $\beta$ CD and the  $\alpha$ HL pore were calculated by using  $K_d = k_{off}/k_{on}$ . Each column represents the mean  $\pm$  s.d. for 10 or more determinations: dark gray, natural amino acids; light gray, unnatural amino acids. Data adapted from Gu and colleagues (15) are marked (\*). (D) Representative current traces from single-channel recordings of  $\beta$ CD binding to M113F<sub>4</sub>M113F<sub>1</sub>F<sub>3</sub> and M113N<sub>4</sub>M113F<sub>1</sub>F<sub>3</sub>.  $\beta$ CD (40  $\mu$ M final) was added to the trans chamber. The broken line indicates zero current. (E) Interaction of  $\beta$ CD with homomeric  $\alpha$ HL pores containing hydrophobic amino acids at position 113.  $K_d$  values for the interaction between  $\beta$ CD and the  $\alpha$ HL pore were calculated by using  $K_d = k_{off}/k_{on}$ . Each column represents the mean  $\pm$  s.d. for ten or more determinations: dark gray, natural amino acids; light gray, unnatural amino acids. Data adapted from Gu and colleagues (15) are marked (\*). (F)  $k_{off}$  values for  $\beta$ CD from heteroheptamers formed with M113F and M113V subunits and with M113N and M113V subunits.  $\beta$ CD (40  $\mu$ M final) was added to the trans chamber. The  $k_{on}$  values for  $\beta$ CD for all these mutants are similar, at  $\sim 3 \times 10^5$  M<sup>-1</sup> s<sup>-1</sup>. Empty square: average  $k_{off}$  values for the mutant (bar is  $\pm$  s.d.). Filled square: M113V<sub>3</sub>M113F<sub>4</sub>; filled circle: M113V<sub>4</sub>M113F<sub>3</sub>; filled upright triangle: M113V<sub>3</sub>M113N<sub>4</sub>; filled inverted triangle: M113V<sub>4</sub>M113N<sub>3</sub>.

(Table S4). In summary, the three groups of tight-binding mutants comprise  $\alpha$ HL pores incorporating, at position 113: (i) the hydrogen-bonding amino acids N, D (the latter would have to be largely in the protonated form), and possibly H; (ii) the aromatics F, Y, f1F, and possibly H, and more weakly W; (iii) the  $\beta$ -branched amino acids V, Cpg. There may be yet other means by which CDs can bind to the  $\alpha$ HL pore. For example, we earlier found that hepta-6-sulfato- $\beta$ CD can bind tightly to  $\alpha$ HL pores containing the

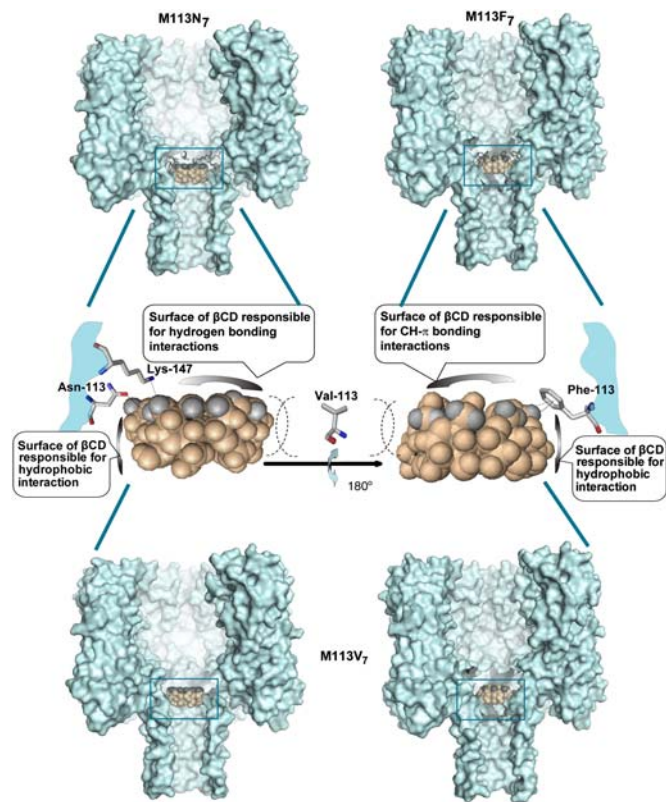
M113Q mutation (37). Presumably, this CD is bound at a site lower down in the  $\beta$  barrel in a fashion that includes hydrogen bonding to the Gln at position 139. While the various mutants exhibited widely different  $k_{off}$  values, the value of  $k_{on}$  was almost invariant and averaged  $\sim 2.3 \times 10^5$  M<sup>-1</sup> s<sup>-1</sup> (Table S2) (15). Apparently, transport to the binding site is rate limiting, through a route unaffected by mutagenesis.

$k_{off}$  increased precipitously with the addition of WT subunits to M113N<sub>7</sub> (Fig. 1C). Crystal structures of M113N<sub>7</sub> show that residues 111, 113, and 147 are reorganized by comparison with WT<sub>7</sub> and then undergo a more limited rearrangement when  $\beta$ CD binds (Fig. S5). For example, the side chain of Lys-147 shifts position to form a bifurcated hydrogen bond with a 3-hydroxyl group of  $\beta$ CD and the side chain carbonyl of an Asn-113 (Fig. S6). Therefore, the side chains of residues 111, 113, and 147 might be in a variety of conformations in WT<sub>7-n</sub>M113N<sub>n</sub> heteromers and offer less well preorganized binding sites for  $\beta$ CD than they do in M113N<sub>7</sub>. Further, the intramolecular hydrogen bonds of the secondary hydroxyls in  $\beta$ CD (38) must be disrupted upon binding as both hydroxyls on each glucose ring form hydrogen bonds to the mutant subunits (Fig. 2B). Because the hydrogen bonds that are broken in  $\beta$ CD are arranged in a circle, the breakage of bonds involving a single glucose (three bonds in all) will be relatively more disruptive than those involving adjoining glucose residues or the entire circle. The overall binding cooperativity in M113N<sub>7</sub> could be attributed to enthalpic cooperativity outweighing entropic penalties to binding (39). Positive cooperativity has been observed previously in fairly rigid model systems (40).

By contrast with M113N<sub>7</sub>, there is little movement of side chains in (M113F)<sub>7</sub> by comparison with WT<sub>7</sub> and little movement, including Phe-113, upon binding  $\beta$ CD (Fig. S7A). Further, the crystal structure of M113F<sub>7</sub>  $\cdot$   $\beta$ CD suggests that each Phe residue interacts independently with the  $\beta$ CD through what appear to be CH- $\pi$  interactions (Fig. S7B). These interactions are expected to be weak and not strongly directional and hence offer less enthalpic cooperativity, as supported by the B-factors (crystallographic temperatures factors) at the primary  $\beta$ CD binding site, which are between  $\sim 40$  and 50. Positive cooperativity is observed, but it is less pronounced than in the case of M113N<sub>7</sub> (Table S5). In the case of M113N<sub>7</sub>, the B-factors of the residues that bind  $\beta$ CD are in the 20s implying that the  $\beta$ CD is more rigidly held than it is in M113F<sub>7</sub>.

The binding of sugars to aromatic residues in proteins can include CH- $\pi$  bonding (41) or OH- $\pi$  bonding or a finely balanced complement of both (42, 43). However, we have dismissed the possibility of an OH- $\pi$  interaction between Phe-113 and the 6-hydroxyl groups of  $\beta$ CD as the distance between the center of the phenyl rings to the nearest hydroxyl oxygen is higher ( $5.2 \pm 0.65$  Å,  $n = 7$ ) than that expected for a favorable OH- $\pi$  interaction (33). While we propose that  $\beta$ CD binds to Phe-113 through a C-6 CH- $\pi$  interaction (Fig. S7B), the distances between the center of the Phe-113 ring and the nearest C-6 of  $\beta$ CD observed in the M113F<sub>7</sub>  $\cdot$   $\beta$ CD structure ( $4.66 \pm 0.24$  Å,  $n = 7$ ) are in the upper range of the expected distance for a strong interaction, which is  $\sim 4.5$  Å (33). The angle between the normal to the aromatic rings and the line connecting the C-6 atoms to the aromatic midpoint is  $8.0 \pm 5.6^\circ$ , which is well within the expected range (44). The measurements with M113f5F<sub>7</sub> argue against a hydrophobic interaction between Phe residues at position 113 and the  $\beta$ CD ring. In f5F, the hydrophobicity of the phenyl ring is significantly increased (45) yet M113f5F<sub>7</sub> binds  $\beta$ CD weakly, like WT<sub>7</sub> (Fig. 3C and Table S24).

By contrast with F, f1F, Y and N, homomeric  $\alpha$ HL pores with f5F and W at position 113 bound  $\beta$ CD relatively weakly (Fig. 3C and Table S24). In the case of f5F, the powerful electron withdrawing action of the five fluorine atoms leaves a highly increased positive charge at the center of the ring (46, 47), mitigating against a hydrogen-bonding interaction. The electron-rich Trp



**Fig. 4.** Molecular model showing the three classes of interaction between the  $\alpha$ HL pore and  $\beta$ CD identified in this work. The model identifies the region of  $\beta$ CD responsible for each interaction (H atoms interacting with Phe-113 or Asn-113 and Lys-147: gray). The first class of interaction is with aromatic residues and involves the seven  $-\text{CH}_2\text{OH}$  groups of the  $\beta$ CD. The second class is typified by the interactions with Asn at position 113, which involve hydrogen-bonds to the secondary 2-hydroxyls of the  $\beta$ CD. Structural studies show that this interaction is supported by hydrogen bonding between Lys-147 and the secondary 3-hydroxyls of the  $\beta$ CD. Structural studies and experiments with heteromers suggest that the  $\beta$ CD in M113F<sub>7</sub> is in the opposite orientation to the  $\beta$ CD in M113N<sub>7</sub>, in support of the model shown here. As the interaction with Val is hydrophobic, it is not directional and  $\beta$ CD may not bind at the same position inside the  $\beta$  barrel as it does in M113F<sub>7</sub> or M113N<sub>7</sub>.

ring (44, 46, 47) should favor hydrogen bonding, but here we cannot make a direct comparison with the crystal structure of M113F<sub>7</sub> as the indole ring is far larger than benzene. It is possible that it cannot become oriented in the same manner and that it is misaligned for hydrogen bonding.

Our experiments suggest that M113V<sub>7</sub> and M113Cp<sub>7</sub> bind  $\beta$ CD in a third way. In heteromers with M113V, both M113F and M113N reduce the affinity of the pore for  $\beta$ CD suggesting that neither the  $\text{CH}-\pi$  interaction with Phe-113 nor the hydrogen-bonding interactions with Asn-113 and Lys-147 are compatible with binding to Val. Close interactions of Val with glucose rings have been noted previously (48). Therefore, we propose that the Val side-chain interacts with the side of the glucose ring. This interaction might occur in one or both orientations of the CD ring (Fig. 4).

## Conclusion

We provide structural information on engineered protein nanopores and describe three distinct ways in which  $\beta$ CD can bind within the lumen of mutant  $\alpha$ HL pores in atomic detail. Our results will be useful in several areas of basic science and biotechnology. By using host molecules lodged within the  $\alpha$ HL pore, host-guest interactions can be investigated in fine detail at the single-molecule level (17, 49). The present work will now permit us to examine binding events at a known face of a CD. The work

also provides information for designing new binding sites within the lumen of the  $\alpha$ HL pore (37) or within other  $\beta$  barrel proteins (21, 50) and for using molecular design to devise ways in which to covalently attach CDs within pores (13, 51). These areas impact practical applications of nanopore technology including stochastic sensing (8), single-molecule DNA sequencing (9, 12, 13, 52), the use of nanoreactors for the observation of single-molecule chemistry (10), and the construction of nano- and microdevices (11, 53), as well as the design of CDs as therapeutic agents (23, 24).

## Methods

Full details of the experimental procedures can be found in *SI Appendix*.

## Materials

L-Amino acids were obtained as follows: 4-fluorophenylalanine (f1F) (Fluka); pentafluorophenylalanine (f5F) (PepTech Corp.); cyclopropylglycine (Cpg) (Tyger); cyclopropylalanine (Cpa) (Tyger). 4-*N*-benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxycytidine-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite and bis(2-cyanoethyl)-N,N-diisopropylphosphoramidite for the synthesis of pdCpA were purchased from Glen Research and Toronto Research Chemicals, respectively.

**Preparation of NVOC-Protected Aminoacyl-pdCpA.** NVOC-protected aminoacyl-pdCpAs were prepared as reported previously by reacting the dinucleotide pdCpA with N-protected, carboxylic acid-activated, amino acids (54–56).

**Preparation of NVOC-Protected Aminoacyl-tRNA.** NVOC-protected aminoacyl-pdCpAs were ligated enzymatically with a truncated tRNA, prepared by using methods described elsewhere (57, 58).

**Genetic Constructs and Mutagenesis.** All new  $\alpha$ HL constructs were verified by DNA sequencing. Details of each construct can be found in *SI Appendix*.

**Synthesis, Assembly, and Purification of Mutant  $\alpha$ HL pores.**  $\alpha$ HL monomers (WT and mutants) were prepared in vitro by coupled transcription and translation (IVTT) and assembled into homoheptamers on rabbit red blood cell membranes followed by purification by SDS-PAGE as described earlier (59). Heteroheptamers were prepared by mixing the two required DNAs (one encoding an  $\alpha$ HL with a D8 tail) before IVTT and then oligomerizing the mixed translation products on rabbit red blood cell membranes. Pores with the desired combinations of subunits were purified by SDS-PAGE (59).

**Synthesis, Assembly, and Purification of  $\alpha$ HL Mutants Containing Unnatural Amino Acids.**  $\alpha$ HL polypeptides containing unnatural amino acids were synthesized by IVTT in the presence of rabbit red blood cell membranes. The plasmid with a stop codon (TAG) at position 113 was used. Deprotected aminoacyl-tRNAs (*SI Appendix*) were added to the IVTT mixtures. For heteroheptamers with subunits containing unnatural amino acids in combination with M113N or M113F, monomers were first made, which were then coassembled on rabbit red blood cell membranes and subsequently purified by SDS-PAGE.

**Single-Channel Current Recordings in Planar Lipid Bilayers.** (15, 60) Recordings were made with 1.0 M NaCl, 10 mM sodium phosphate, pH 7.5, in both chambers, at an applied potential of +40 mV. Data were recorded at  $22 \pm 2^\circ\text{C}$ . The bilayer was formed from 1,2-diphytanoyl-*sn*-glycero-phosphocholine (Avanti Polar Lipids). Proteins were added to the cis chamber, and  $\beta$ CD to the trans chamber. Single-channel currents were recorded with an Axopatch 200B patch-clamp amplifier (Axon Instruments) and filtered at 2 kHz with a built-in 4-pole low-pass Bessel Filter. The data were acquired at a sampling rate of 10 kHz. For mutants that bind  $\beta$ CD strongly, the data were acquired for at least 30 min and for weak-binding mutants for at least 10 min.

**Kinetic Data Analysis.** Current amplitude and dwell-time histograms were made by using ClampFit 9.0. The mean dwell times,  $\tau_{\text{off}}$ , were determined by fitting the dwell-time histograms to single exponentials. Values of  $k_{\text{on}}$  and  $k_{\text{off}}$  were obtained by using the mean dwell times and mean interevent intervals, as described previously (15, 60). This analysis assumes a binary interaction, which was supported in all cases examined by the finding of only one major blockade level and a single exponential distribution of dwell times ( $\tau_{\text{off}}$ ).



**Protein Crystallography.** Details can be found in [SI Appendix](#). Protein Data Bank: The coordinates and structure factors of the described structures have been deposited with accession codes 3M2L (M113F<sub>7</sub>), 3M3R (M113F<sub>7</sub> ·  $\beta$ CD), 3M4D (M113N<sub>7</sub>), 3M4E (M113N<sub>7</sub> ·  $\beta$ CD).

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